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24,25,28-Trihydroxyvitamin D_2 and 24,25,26-Trihydroxyvitamin D_2: Novel Metabolites of Vitamin D_2[†]

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ABSTRACT: Understanding of the inactivation pathways of 25-hydroxyvitamin D_2 and 24-hydroxyvitamin D_2, the two physiologically significant monohydroxylated metabolites of vitamin D_2, is of importance, especially during hypervitaminosis D_2. In a recent study, it has been demonstrated that the inactivation of 24-hydroxyvitamin D_2 occurs through its conversion into 24,26-dihydroxyvitamin D_2 [Koszewski, N. J., Reinhardt, T. A., Napoli, J. L., Beitz, C. D., & Horst, R. L. (1988) *Biochemistry 27*, 5785]. At present, little information is available regarding the inactivation pathway of 25-hydroxyvitamin D_2 except its further metabolism into 24,25-dihydroxyvitamin D_2 [Jones, G., Rosenthal, A., Segev, D., Mazur, Y., Frolow, F., Halfon, Y., Rabinovich, D., & Shakked, Z. (1979) *Biochemistry 18*, 1094]. In our present study we investigated the metabolic fate of 25-hydroxyvitamin D_2 in the isolated perfused rat kidney and demonstrated its conversion not only into 24,25-dihydroxyvitamin D_2 but also into two other new metabolites, namely, 24,25,28-trihydroxyvitamin D_2 and 24,25,26-trihydroxyvitamin D_2. The structure identification of the new metabolites was established by the techniques of ultraviolet absorption spectrophotometry and mass spectrometry and by the characteristic nature of each new metabolite's susceptibility to sodium metaperiodate oxidation. In order to demonstrate the physiological significance of the two new trihydroxy metabolites of vitamin D_2, we induced hypervitaminosis D_2 in a rat using [1α-^{3}H]vitamin D_2 and analyzed its plasma for the various [1α-^{3}H]vitamin D_2 metabolites on two different high-pressure liquid chromatography systems. The results indicate that both 24,25,28-trihydroxyvitamin D_2 and 24,25,26-trihydroxyvitamin D_2 circulate in the vitamin D_2 intoxicated rat in significant amounts along with other previously identified monohydroxy and dihydroxy metabolites of vitamin D_2, namely, 24-hydroxyvitamin D_2, 25-hydroxyvitamin D_2, and 24,25-dihydroxyvitamin D_2. Thus, it may be hypothesized that the two new trihydroxy metabolites of vitamin D_2 play an important physiological role in the deactivation of 25-hydroxyvitamin D_2, especially during hypervitaminosis D_2.

Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are the two important nutritional forms of vitamin D out of all the known forms of vitamin D (Napoli et al., 1979). Provitamin D_3 (7-dehydrocholesterol) exists in the skin of animals, while provitamin D_2 (ergosterol) exists in plants and in many lower organisms such as fungi and yeasts. Provitamins D_2 and D_3 are converted into their corresponding vitamins D_2 and D_3 when exposed to UV radiation. The two vitamins differ only in the structure of their side chains (the side chain of vitamin D_2 has an extra methyl group at C-24 and a double bond between C-22 and C-23 when compared to the side chain of vitamin D_3). Historically, vitamin D_2 became important in clinical medicine as it was the first synthetic vitamin D preparation available for the treatment of rickets, and it is still being widely used to satisfy both therapeutic and nutritional needs of man and other commercially important mammals.

At present, it is the general belief that the further metabolic pathways of vitamin D_2 are similar to those of vitamin D_3 (Norman et al., 1982). Vitamin D_2, like vitamin D_3, undergoes hydroxylations at C-25 in liver and at C-1 in kidney to form $1,25(OH)_2D_2$,[1] the hormonally active form of vitamin D_2 (Jones et al., 1975). During the past decade, the pathways of side-chain metabolism of vitamin D_3 metabolites [25-OH-D_3 and $1,25(OH)_2D_3$] have been studied extensively. It is now apparent that the side chains of both 25-OH-D_3 and $1,25(OH)_2D_3$ undergo analogous metabolic alterations resulting in the formation of many relatively inactive metabolites, and this subject has been extensively studied in several laboratories and was reviewed recently by Jones et al. (1987). Because of the obvious structural differences between the side chains

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[1] Abbreviations: 24-OH-D_2, 24-hydroxyvitamin D_2; 25-OH-D_2, 25-hydroxyvitamin D_2; $24,25(OH)_2D_2$, 24(R),25-dihydroxyvitamin D_2; $24,26(OH)_2D_2$, 24,26-dihydroxyvitamin D_2; $25,26(OH)_2D_2$, 25,26-dihydroxyvitamin D_2; $25,28(OH)_2D_2$, 25,28-dihydroxyvitamin D_2; $24,25,28(OH)_3D_2$, 24,25,28-trihydroxyvitamin D_2; $24,25,26(OH)_3D_2$, 24,25,26-trihydroxyvitamin D_2; $1,25(OH)_2D_2$, 1,25-dihydroxyvitamin D_2; $1,24,25(OH)_3D_2$, 1,24,25-trihydroxyvitamin D_2; $1,24,25,28(OH)_4D_2$, 1,24,25,28-tetrahydroxyvitamin D_2; $1,24,25,26(OH)_4D_2$, 1,24,25,26-tetrahydroxyvitamin D_2; 24-keto-25,26,27-trinor-D_3, 24-keto-25,26,27-trinorvitamin D_3; 25-OH-24-keto-26-nor-D_3, 25-hydroxy-24-keto-26-norvitamin D_3; 24-OH-25-keto-26-nor-D_3, 24-hydroxy-25-keto-26-norvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; $25,26(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; HPLC, high-performance liquid chromatography.

EXHIBIT B

of vitamins D_2 and D_3, it appeared logical to find different pathways of side-chain metabolism for vitamin D_2 metabolites [25-OH-D_2 and 1,25(OH)$_2D_2$]. Accordingly, a novel metabolic pathway for 1,25(OH)$_2D_2$ has been recently described, and it has become obvious that the side-chain metabolism of 1,25(OH)$_2D_2$ differs from that of 1,25(OH)$_2D_3$ and that the differences become apparent only after the C-24 hydroxylation step (Horst et al., 1986; Reddy & Tserng, 1986). Following the C-24 hydroxylation step, 1,25(OH)$_2D_2$ undergoes hydroxylations either at C-28 to form 1,24,25,28(OH)$_4D_2$ or at C-26 to form 1,24,25,26(OH)$_4D_2$ (Reddy & Tserng, 1986). Even though a complete functional evaluation of the two new metabolites of 1,25(OH)$_2D_2$ has not been performed yet, it appears from our preliminary results that the new pathway of side-chain metabolism of 1,25(OH)$_2D_2$ is indeed a pathway intended to inactivate 1,25(OH)$_2D_2$ (G. S. Reddy and R. L. Horst, unpublished observations). At present, very little is known regarding the inactivation pathway of 25-OH-D_2 except its conversion into 24(R),25(OH)$_2D_2$ (Jones et al., 1979, 1980). We predicted the inactivation of 25-OH-D_2 through its conversion into 24,25,28(OH)$_3D_2$ and 24,25,26(OH)$_3D_2$ in a manner analogous to the inactivation of 1,25(OH)$_2D_2$ through its conversion into 1,24,25,28(OH)$_4D_2$ and 1,24,25,26(OH)$_4D_2$. Therefore, this study was performed with the aim to identify 24,25,28(OH)$_3D_2$ and 24,25,26(OH)$_3D_2$ as the further metabolites of 25-OH-D_2 and to demonstrate their formation especially during hypervitaminosis D_2.

Materials and Methods

General. Ultraviolet absorbance spectra were taken in 2-propanol with a Beckman DU-8 recording spectrophotometer. High-performance liquid chromatography (HPLC) was performed with a Waters Model 6000 equipped with a detector (Model 440) to monitor UV-absorbing material at 254 nm (Waters Associates, Milford, MA). All HPLC solvents were purchased from Burdick & Jackson Laboratories, Muskegon, MI. Mass spectra (70 eV) were obtained on a Hewlett-Packard 5985 B mass spectrometer. Samples of metabolites (0.5 μg each) were introduced into the ion source maintained at 200 °C via a direct-insertion probe.

Vitamin D Compounds. Vitamin D_2 was purchased from Sigma Chemical Co. (St. Louis, MO). 25-OH-D_2 was a gift from Drs. J. A. Campbell and J. Babcock (Upjohn Co., Kalamazoo, MI). 24(R),25(OH)$_2D_2$ was a gift from Dr. T. Kobayashi, Kobe Women's College of Pharmacy, Kobe, Japan. All the various synthetic standards of both vitamins D_2 and D_3 metabolites used in this study were a gift from Drs. Milan Uskokovic and E. G. Baggiolini (Hoffmann-La Roche Inc., Nutley, NJ). Authentic 24-OH-D_2 was isolated from the serum of vitamin D_2 intoxicated rats, and the structure of biologically produced 24-OH-D_2 was verified by mass spectrometry as described before (Jones et al., 1980). [26-^{3}H]-Vitamin D_2 (1.2 Ci/mmol) was a gift from Dr. J. L. Napoli (State University of New York, Buffalo, NY).

Animals. Male Sprague-Dawley rats (about 300 g), purchased from Zivic Miller Laboratories, Inc., Allison Park, PA, were fed a regular rodent diet (Agway, Syracuse, NY), containing 0.97% calcium, 0.85% phosphorus, and vitamin D, 1045 IU/kg.

Study of 25-OH-D_2 Metabolism in the Kidneys of Vitamin D_2 Intoxicated Rats Using the Technique of Kidney Perfusion. Kidney perfusions were performed as described before in detail (Reddy et al., 1982, 1983). In order to produce the various further metabolites of 25-OH-D_2 in quantities sufficient for their structural identification, we performed two kidney perfusions. It has been demonstrated ... (1984) that the activity of the enzymes involved in the further metabolism of 25-OH-D_3 and 24,25(OH)$_2D_3$ can be induced in the kidneys by intoxicating the rats with vitamin D_3. Therefore, in these perfusion experiments, we induced hypervitaminosis D_2 in rats in order to increase the activity of the renal enzymes that are involved in the further metabolism of 25-OH-D_2. On the basis of the information given in a previous study by Shepard and DeLuca (1980), we first estimated 1000 μg of vitamin D_2 as a safe total dose that can intoxicate a rat without causing death. We then induced hypervitaminosis D_2 in each rat by administering 100 μg of vitamin D_2 in 100 μL of 95% ethanol intramuscularly each day for a period of 10 days. The last dose of vitamin D_2 was administered to the rat 24 h prior to the isolation of the kidney. Each kidney isolated from a vitamin D_2 intoxicated rat was perfused for 4 h with cold 25-OH-D_2 (200 nmol in 100 μL of ethanol) which was introduced into 100 mL of perfusate after a 5-min stabilization period following the isolation of the kidney. In this study, we also performed a control perfusion experiment in the absence of a kidney and demonstrated that there was no metabolism of 25-OH-D_2 in the perfusion apparatus in the absence of a kidney (data not shown).

Lipid Extraction. Lipid extraction of the kidney perfusate was performed according to the procedure of Bligh and Dyer (1959), except that methylene chloride was substituted for chloroform.

Isolation and Purification of the Various Metabolites of 25-OH-D_2 from Kidney Perfusate for Their Structure Identification. Bulk lipid extract obtained from 180 mL of the final perfusate belonging to the two kidney perfusions was divided into nine portions. Each lipid portion was then subjected directly to HPLC under the chromatographic conditions described in the legend to Figure 1 without overloading the column with lipid. Even though radiolabeled 25-OH-D_2 was not available to us at the time of our present study, we were able to trace the various metabolites of 25-OH-D_2 by monitoring their UV absorbance at 254 nm. The elution volume of each UV peak during the first HPLC system (shown in Figure 1, lower panel) was as follows: peak 1, 40–46 mL; peak 2, 88–92 mL; peak 3, 92–96 mL; peak 4, 104–108 mL. All the selected UV peaks obtained during the first nine HPLC runs were pooled, and each individual UV peak was then subjected to a second HPLC system with the same Zorbax-SIL column (4.6 mm × 25 cm) eluted with methylene chloride/2-propanol (94:6) at a flow rate of 2 mL/min. The elution volume of each UV peak during the second HPLC system was as follows: peak 1, 12–14 mL; peak 2, 30–34 mL; peak 3, 24–28 mL; peak 4, 40–48 mL. Each UV peak obtained from the second HPLC system was rechromatographed twice with the first HPLC run. At this time, the purity of each UV peak was tested by obtaining their UV spectra. The UV-absorbing material from peaks 1, 2, and 4 exhibited a UV spectrum that is characteristic for all D vitamins. The UV-absorbing material from peak 3 exhibited a UV spectrum with a UV_{max} at 243 nm and thus did not exhibit a UV spectrum characteristic for D vitamins. All three vitamin D_2 metabolites obtained from peaks 1, 2, and 4 were then subjected to sodium metaperiodate oxidation and mass spectrometry in order to identify their chemical structures.

Sodium Metaperiodate Oxidation of 24,25(OH)$_2D_2$ and the Two New Metabolites of 25-OH-D_2. The susceptibility of 24,25(OH)$_2D_2$ and the two new metabolites of 25-OH-D_2 to sodium metaperiodate ($NaIO_4$) oxidation was tested in order to locate the exact positions of the hydroxyl groups in each

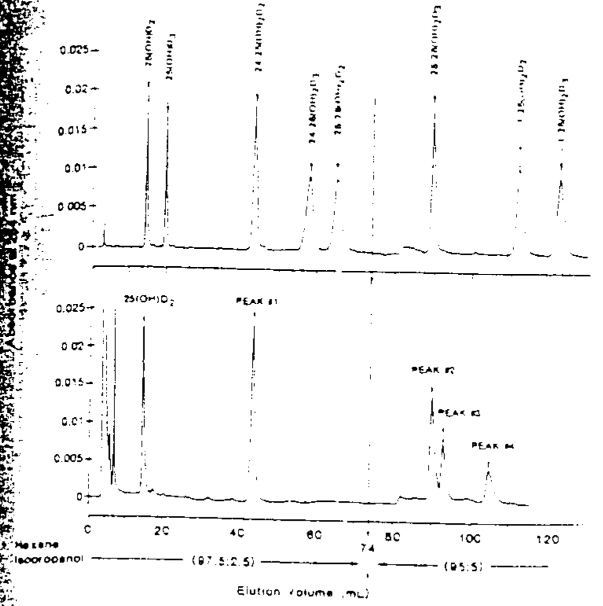

FIGURE 1: HPLC profiles of a mixture of various authentic synthetic metabolites of vitamins D_2 and D_3 (upper panel) and the lipid extract obtained from 20 mL of perfusate (lower panel). HPLC in both cases was performed on a Zorbax-Sil column (25 cm × 4.6 mm) that was first eluted with hexane–2-propanol (97.5:2.5) at a flow rate of 2 mL/min until 25,28(OH)$_2$D$_2$ was eluted out of the column. Then, the first solvent system was changed to a second solvent system (hexane–2-propanol, 95:5), keeping the flow rate same to elute the more polar metabolites of vitamin D_2 out of the column. The various metabolites of 25-OH-D_2 were identified by monitoring their UV absorbance at 254 nm. Peaks 1, 2, and 4 represent 24,25(OH)$_2$D$_2$, 24,25,28(OH)$_3$D$_2$, and 24,25,26(OH)$_3$D$_2$, respectively. Peak 3 represents a non vitamin D contaminant produced by the kidney. Note that the size of the 25-OH-D_2 peak shown in the lower panel of the figure represents only $^1/_{10}$ that of the original peak.

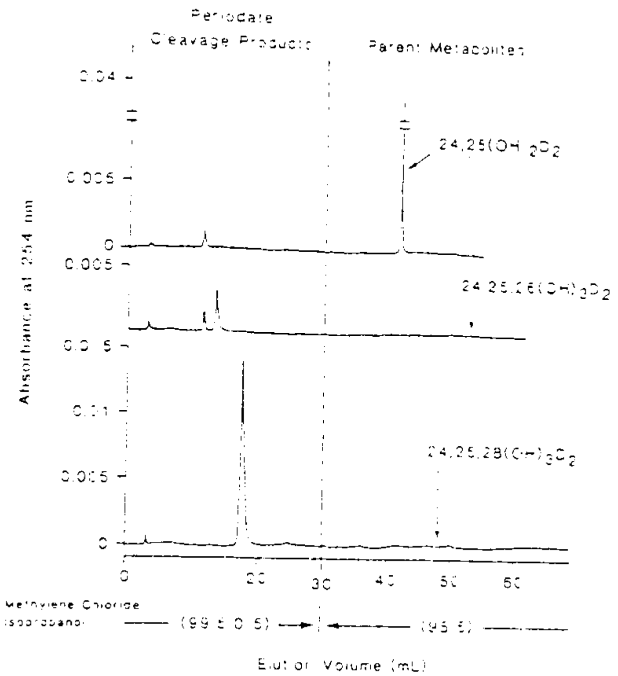

FIGURE 2: HPLC analysis of the reaction products, obtained by treating 0.3–0.5 μg of each metabolite of 25-OH-D_2 with sodium metaperiodate for 5 min: 24,25(OH)$_2$D$_2$ (upper panel); 24,25,26-(OH)$_3$D$_2$ (middle panel); 24,25,28(OH)$_3$D$_2$ (lower panel). HPLC was performed on a Zorbax-Sil column (25 cm × 4.6 mm). The column was first eluted with methylene chloride–2-propanol (99.5:0.5) at a flow rate of 2 mL/min until the periodate cleavage product(s) of each metabolite eluted out of the column. Then, the solvent system was switched to methylene chloride–2-propanol (95:5) at the same flow rate to elute the unreacted parent metabolites. Arrows indicate the elution position of the parent metabolites.

between two carbons when either both carbons bear hydroxyl groups or one carbon bears a hydroxyl group and the other bears a keto group. Each metabolite (0.3–0.5 μg each) was dissolved in 15 μL of methanol and was allowed to react with 10 μL of 5% aqueous $NaIO_4$ for 5 min. All the reactions were carried out at room temperature (25 °C). The appropriate HPLC systems used to isolate the periodate cleavage products of all three metabolites are described in detail in the legend to Figure 2. Even though all three metabolites of 25-OH-D_2 isolated from the kidney perfusate were susceptible to periodate oxidation, we noticed that during an incubation period of 5 min only 5% of 24,25(OH)$_2$D$_2$ was converted into its corresponding periodate cleavage product, whereas both 24,25,28-(OH)$_3$D$_2$ and 24,25,26(OH)$_3$D$_2$ were completely converted into their corresponding periodate cleavage products (Figure 2). In order to produce the periodate cleavage product of 24,25(OH)$_2$D$_2$ in sufficient quantity, we had to increase the incubation period to 1 h (data not shown). The reason for the differences in the degree of susceptibility to periodate oxidation between the three metabolites of 25-OH-D_2 is as follows. The vicinal diol at C-24 and C-25 in 24,25(OH)$_2$D$_2$ is not readily susceptible to periodate oxidation as it is sterically hindered by C-28, C-27, and C-26 methyl groups. This phenomenon was also noticed by Jones et al. (1987). However, the vicinal diol at C-24 and C-25 in 24,25,28(OH)$_3$D$_2$ and the vicinal diol at C-25 and C-26 in 24,25,26(OH)$_3$D$_2$ are not sterically hindered and, hence, are mainly susceptible to periodate oxidation.

Study of in Vivo Metabolites of Vitamin D_2 in a Vitamin D_2 Intoxicated Rat. This experiment was designed to demonstrate the in vivo existence of two new trihydroxy metabolites of vitamin D_2 in hypervitaminosis D_2. Because of a limited supply of [3α-^{3}H]vitamin D_2, we only performed this experiment in a single rat. Hypervitaminosis D_2 was induced in the rat with the same dose of vitamin D_2 and the method that has been used in our present study during the investigation of in vitro metabolism of 25-OH-D_2 in kidneys isolated from vitamin D_2 intoxicated rats. We first prepared a mixture of nonradioactive vitamin D_2 (1000 μg) and [3α-^{3}H]vitamin D_2 (20 μCi) in 1 mL of 95% ethanol and thus obtained a specific activity of 20 cpm/1 ng of vitamin D_2. The rat received 100 μL of the above mixture intramuscularly each day over a period of 10 days. Twenty four hours following the final dose, the rat was sacrificed by exsanguination to obtain 6 mL of plasma, which was divided into two portions. The first 3 mL portion was again divided into three 1-mL portions. All four plasma samples were extracted, and the lipid extract of each sample was subjected to HPLC directly. We first performed preliminary HPLC runs of the lipid obtained from 1-mL plasma samples using the HPLC system described in Figure 1. On the basis of the information obtained from the three preliminary HPLC runs, we developed the HPLC system described in the legend to Figure 5 and measured the various [^{3}H]vitamin D_2 metabolites present in the final 3-mL plasma sample.

Results

Metabolism of 25-OH-D_2 by the Perfused Kidney Isolated from Vitamin D_2 Intoxicated Rats. The lipid concentrate obtained from 20 mL of perfusate was analyzed in a single HPLC run on a straight phase HPLC system (Figure 1, upper panel) that is capable of resolving most of the known metabolites of both 25-OH-D_2 and 25-OH-D_3. From the HPLC

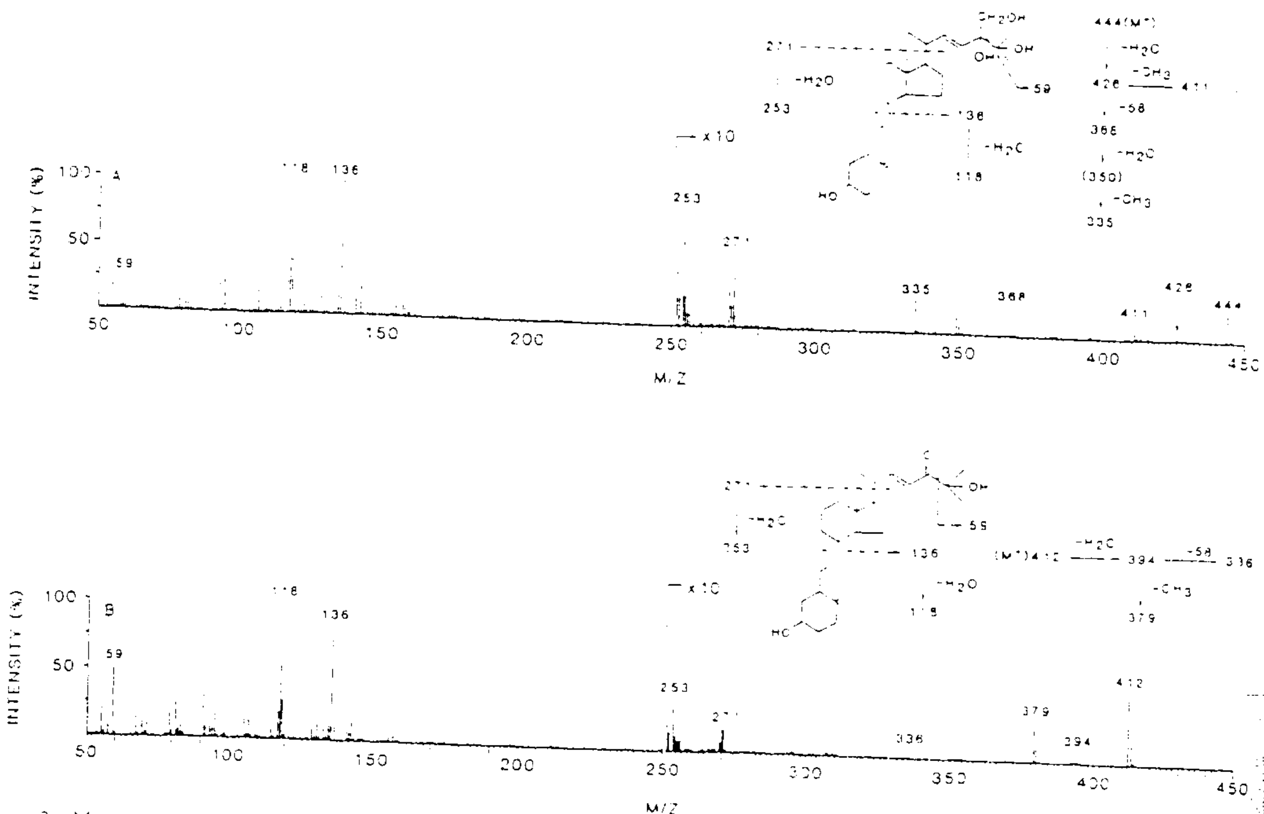

FIGURE 3: Mass spectra of 24,25,28(OH)$_3$-D$_2$ (A) and its periodate cleavage product (B).

chromatogram in Figure 1 (lower panel), it became apparent that there were only four UV peaks (peaks 1–4) following the UV peak of 25-OH-D$_2$. Out of the four UV peaks, UV peak 3 was found to be a non vitamin D lipid contaminant. UV peak 1 comigrated with the synthetic standard of 24(*R*),25-(OH)$_2$D$_2$ on two different HPLC systems and exhibited a mass spectrum identical with the one described previously by Jones et al. (1979, 1980) (data not shown). Also, the metabolite was susceptible to periodate oxidation and resulted in the formation of 24-keto-25,26,27-trinor-D$_2$, the expected periodate cleavage product of 24,25(OH)$_2$D$_2$. The mass spectrum of 24-keto-25,26,27-trinor-D$_2$ was identical with the one previously described by Jones et al. (1979) (data not shown). Thus, on the basis of the periodate oxidation and the mass spectrometric data, the metabolite of 25-OH-D$_2$ in UV peak 1 was identified as 24,25(OH)$_2$D$_2$. The metabolite of 25-OH-D$_2$ in UV peak 2 comigrated with the synthetic standard of 25,26-(OH)$_2$D$_3$ (Figure 1) and was later identified as 24,25,28-(OH)$_3$D$_2$. The metabolite of 25-OH-D$_2$ in UV peak 4 migrated just before the synthetic standard of 1,25(OH)$_2$D$_2$ (Figure 1) and was later identified as 24,25,26(OH)$_3$D$_2$. Thus, the results of our study indicated that 25-OH-D$_2$ was metabolized in the isolated perfused rat kidney into three major metabolites of vitamin D$_2$ out of which 24,25(OH)$_2$D$_2$ was described originally by Jones et al. (1979, 1980) and the remaining two metabolites were found to be new. The detailed description of the structural identification of the two new metabolites of 25-OH-D$_2$ as 24,25,28(OH)$_3$D$_2$ and 24,25,26-(OH)$_3$D$_2$ is as follows.

Structural Identification of 24,25,28(OH)$_3$D$_2$ and 24,25,26(OH)$_3$D$_2$. The two new metabolites of 25-OH-D$_2$ purified from the kidney perfusate exhibited UV spectra with an absorbance maximum at 264 nm and an absorbance minimum at 228 nm. This finding indicated that the two metabolites contained an intact 5,6-cis-triene chromophore (data not shown). The mass spectra (Figures 3A and 4A) of the new metabolites exhibited peaks at m/z 271, 253, 136, and 118. Collectively, the peaks indicated that the secosteroid nucleus of their parent 25-OH-D$_2$ has remained unchanged and that the two new metabolites were formed as a result of changes occurring only on their side chains. The molecular ion at m/z 444 (M$^+$) in the mass spectrum of each new metabolite indicated that both new metabolites contained two additional hydroxyl groups when compared to 25-OH-D$_2$. As we had already determined that the secosteroid nucleus of both new metabolites was intact and similar to the one present in 25-OH-D$_2$, it was possible to conclude that each new metabolite was formed as a result of addition of two hydroxyl groups to the side chain of 25-OH-D$_2$. The exact locations of the two additional hydroxyl groups on the side chain of each individual new metabolite were determined in the following way.

The mass spectrum (Figure 3A) of the new metabolite in UV peak 2 in Figure 1 exhibited a peak at m/z 59 and a peak at m/z 368 formed as a result of elimination of 58 mass units from the peak at m/z 426 (McLafferty type rearrangement). This finding indicated that the metabolite contained an intact C-25 hydroxyl group with no hydroxylations occurring on C-26 and C-27. Also, this metabolite was susceptible to periodate oxidation, and the mass spectrum of the periodate cleavage product (Figure 3B) showed a molecular ion at m/z 412 which indicated that the parent compound had lost CH$_3$OH (32 mass units) during the process of periodate oxidation. The characteristic peak at m/z 59 and a peak at m/z 336 formed as a result of elimination of 58 mass units from the peak at m/z 394 (McLafferty type rearrangement) indicated that the periodate cleavage product still contained an intact C-25 hydroxyl group like its parent metabolite. On the basis of this information the periodate cleavage product was identified as 25-OH-24-keto-28-nor-D$_2$. We also observed that 25-OH-24-keto-28-nor-D$_2$ was further susceptible to periodate oxi-

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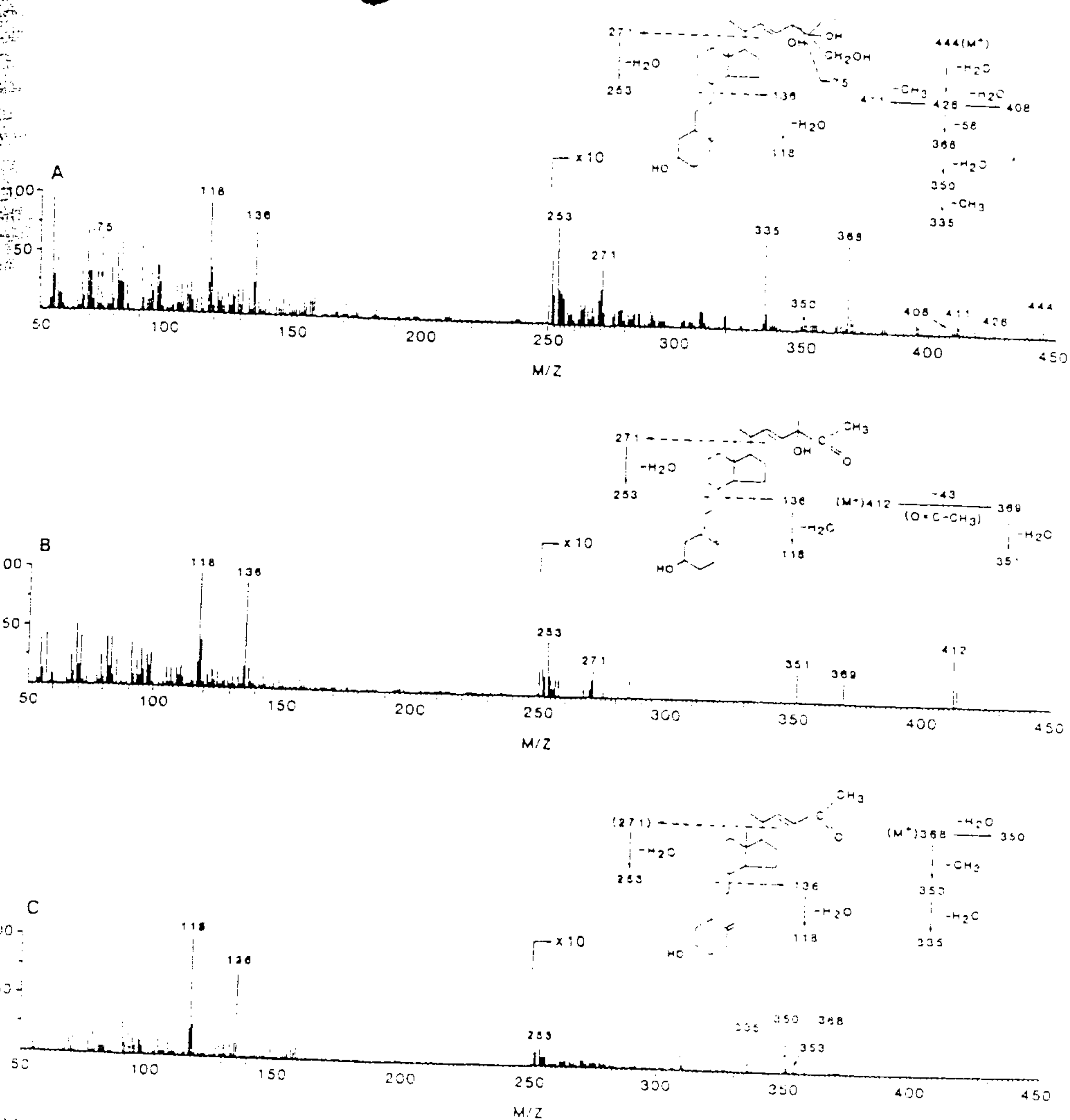

FIGURE 4: Mass spectra of 24,25,26(OH)$_3$D$_2$ (A) and its two periodate cleavage products (B and C).

dation, and this finding provided indirect evidence for the presence of a vicinal diol (keto group at C-24 and hydroxyl group at C-25) in 25-OH-24-keto-28-nor-D$_2$. However, we could not isolate the C-24 acid, the expected periodate cleavage product of 25-OH-24-keto-28-nor-D$_2$, as the HPLC systems developed in our laboratory at the time of this study were not designed to isolate highly polar acids. The formation of 25-OH-24-keto-28-nor-D$_2$ from the new trihydroxy metabolite of vitamin D$_2$ as a result of periodate oxidation would be possible only if the new metabolite contained vicinal hydroxyl groups at C-24 and C-28. Thus, it was finally concluded that the new trihydroxy metabolite of vitamin D$_2$ possessed hydroxyl groups at C-24 and C-28 in addition to the original hydroxyl group at C-25, present in its parent 25-OH-D$_2$, and was therefore identified as 24,25,28(OH)$_3$D$_2$.

The mass spectrum (Figure 4A) of the metabolite in UV peak 4 in Figure 1 exhibited a peak at m/z 75 which suggested that the metabolite contained an intact C-25 hydroxyl group with an extra hydroxyl group at C-26. Other more convincing evidence of C-26 hydroxylation came due to the presence of characteristic mass fragments at m/z 368, 350, and 335. These mass fragments were produced as a result of McLafferty type rearrangement of an α-substituted β-hydroxy aldehyde resulting from the dehydration of the molecular ion (M$^+$), followed by a loss of CH_3CH_2CHO (58 mass units). This phenomenon is similar to the characteristic decomposition pathway described for α-substituted β-hydroxy esters (Budzikiewicz et al., 1967). Furthermore, this new metabolite was also susceptible to periodate oxidation and gave rise to two cleavage products (Figure 2). The mass spectrum of the more polar periodate cleavage product (Figure 4B) showed a molecular ion at m/z 412 which indicated that the new metabolite had lost CH_3OH (32 mass units) during the process of periodate oxidation. Also, the characteristic loss of $CH_3—C{=}O$ (43 mass units) from the molecular ion gave rise to the peak at m/z 369. With this information, the more polar periodate cleavage product was identified as 24-OH-25-keto-26-nor-D$_2$, the formation of which would only be possible due to the presence of vicinal hydroxyl groups at C-25 and C-26. The less polar periodate cleavage product had exhibited identical

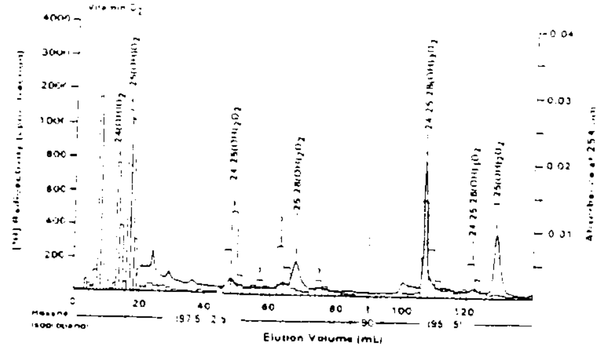

FIGURE 5: HPLC profile of the lipid extract of a plasma sample (3 mL) obtained from a rat given 1000 μg of [^{3}H]vitamin D_2 in divided doses over a period of 10 days. The plasma sample was mixed with authentic nonradioactive vitamin D_2 metabolites [24-OH-D_2, 0.2 μg; 25-OH-D_2, 0.2 μg; 25,28(OH)$_2D_2$, 0.2 μg; 24,25,28(OH)$_3D_2$, 0.5 μg; 1,25(OH)$_2D_2$, 0.5 μg] with the aim of identifying each individual radioactive vitamin D_2 metabolite by its comigration with its corresponding authentic nonradioactive vitamin D_2 metabolite standard. The lipid extract of the plasma sample containing the various [^{3}H]vitamin D_2 metabolites and the authentic nonradioactive vitamin D_2 metabolites was analyzed by HPLC under the chromatographic conditions described in the legend to Figure 1 except that the first solvent system was changed to the second solvent system at an elution volume of 90 mL. The elution positions of the various nonradioactive authentic standards of vitamin D_2 metabolites as monitored by their UV absorbance at 254 nm are depicted in the figure above by the solid line. Note that the UV absorbance profile during the first 10-min HPLC run is not shown for the sake of clarity. Fractions of 1 mL during the first 10 min of the HPLC run and fractions of 2 mL during the remaining period of the HPLC run were collected. The HPLC effluent in each fraction was divided into two equal portions. The first portion was used to measure the radioactivity, which is depicted in the figure by a histogram. The second portion was used for rechromatography of each major metabolite of vitamin D_2 on a second HPLC run using a methylene chloride–2-propanol mixture as the solvent system (data not shown). Thus, we confirmed the identity of each major metabolite of vitamin D_2 by its comigration with its corresponding authentic cold standard on two different HPLC systems.

chromatographic mobility as that of the periodate cleavage product of 24,25(OH)$_2D_3$ (Figure 2). Its mass spectrum (Figure 4C) was identical with the mass spectrum of the periodate cleavage product of 24,25(OH)$_2D_3$, previously published by Jones et al. (1979). With this information, the less polar cleavage product was identified as 24-keto-25,26,27-trinor-D_2. The formation of 24-keto-25,26,27-trinor-D_2 from the new trihydroxy metabolite of vitamin D_2 as a result of periodate oxidation would only be possible if the new metabolite contained vicinal hydroxyl groups at C-24 and C-25. Thus, by putting together the aforementioned data, it was finally concluded that the new trihydroxy metabolite of vitamin D_2 possessed hydroxyl groups at C-24 and C-26 in addition to the original hydroxyl group at C-25, present in its parent 25-OH-D_2, and was therefore identified as 24,25,26-(OH)$_3D_2$.

Identification of both 24,25,28(OH)$_3D_2$ and 24,25,26-(OH)$_3D_2$ as the in Vivo Metabolites in a Vitamin D_2 Intoxicated Rat. From the HPLC chromatogram shown in Figure 5, it became obvious that there were several circulating [^{3}H]vitamin D_2 metabolites in the plasma of a vitamin D_2 intoxicated rat. We were able to estimate the concentration of each vitamin D_2 metabolite as we knew the specific activity of [^{3}H]vitamin D_2 that was administered to the rat. The mean value of each metabolite concentration in 1 mL of plasma estimated from two different HPLC runs was as follows: vitamin D_2, 138 ng; 24-OH-D_2, 39 ng; 25-OH-D_2, 90 ng; 24,25(OH)$_2D_2$, 28 ng; 24,25,28(OH)$_3D_2$, 31 ng; 24,25,26-(OH)$_3D_2$, 10 ng. Thus, from the results of our carefully performed HPLC analysis of the plasma of the vitamin D_2 intoxicated rat, we established both 24,25,28(OH)$_3D_2$ and 24,25,26(OH)$_3D_2$ as the significant in vivo metabolites in hypervitaminosis D_2. Also, our finding of both 24-OH-D_2 and 24,25(OH)$_2D_2$ as the major circulating metabolites in hypervitaminosis D_2 was not surprising in light of a recent study by Koszewski et al. (1988). We also noted in Figure 5 that there were one major and two minor radioactive vitamin D_2 metabolite peaks that migrated just before and after the authentic synthetic standard of 25,28(OH)$_2D_2$. Even though we did not establish the identity of these metabolite peaks, it could be predicted that one of them, especially the major one, might be 24,26(OH)$_2D_2$, a metabolite that was recently identified by Koszewski et al. (1988) as one of the major circulating metabolites of vitamin D_2 in rats intoxicated with vitamin D_2.

Discussion

This paper reports the identification of two new metabolites of 25-OH-D_2 produced in a mammalian kidney. They were identified as 24,25,28(OH)$_3D_2$ and 24,25,26(OH)$_3D_2$. The process of structural identification of the two new metabolites of 25-OH-D_2 was identical with the one described in our previous study (Reddy & Tserng, 1986) for the two analogous metabolites of 1,25(OH)$_2D_2$, namely, 1,24,25,28(OH)$_4D_2$ and 1,24,25,26(OH)$_4D_2$. In this study, we only demonstrated the formation of the new metabolites of 25-OH-D_2 by perfusing rat kidneys with a pharmacological concentration of 25-OH-D_2 (2×10^{-6} M) because of the unavailability of radiolabeled 25-OH-D_2 at the time of this study. However, in a collaborative study that followed our present study, we first synthesized [3α-^{3}H]-25-OH-D_2 enzymatically by perfusing livers isolated from vitamin D deficient rats with [3α-^{3}H]vitamin D_2. We then demonstrated both 24,25,28(OH)$_3D_2$ and 24,25,26(OH)$_3D_2$ as the physiological metabolites of 25-OH-D_2 by perfusing kidneys isolated from normal rats on a regular rodent diet with a physiological concentration of [3α-^{3}H]-25-OH-D_2 (6×10^{-9} M) (G. S. Reddy, R. Fay, and M. F. Holick, unpublished observations). At the present time, we are unable to assess the biological activity of both 24,25,28-(OH)$_3D_2$ and 24,25,26(OH)$_3D_2$ due to the unavailability of these new metabolites in a quantity sufficient for the standard bioassays measuring intestinal calcium transport and bone calcium mobilization. However, as an alternative, it is still possible for us to predict that both 24,25,28(OH)$_3D_2$ and 24,25,26(OH)$_3D_2$ are probably the inactive metabolites of 25-OH-D_2, as we have recently found that the two 1α-hydroxylated metabolites of 24,25,28(OH)$_3D_2$ and 24,25,26-(OH)$_3D_2$ [1,24,25,28(OH)$_4D_2$ and 1,24,25,26(OH)$_4D_2$] are indeed completely inactive in terms of intestinal calcium transport and bone calcium mobilization (G. S. Reddy and R. L. Horst, unpublished observations). Furthermore, in our present study we have demonstrated that both 24,25,28-(OH)$_3D_2$ and 24,25,26(OH)$_3D_2$ are circulating in significant amounts in a vitamin D_2 intoxicated rat. Thus, even though we have not performed a detailed functional evaluation of the two new trihydroxy metabolites of vitamin D_2, it appears logical at the present time to assume that the formation of new metabolites may play an important physiological role in the deactivation of 25-OH-D_2, especially during hypervitaminosis D_2.

In this study, we also considered the possibility of the metabolism of 25-OH-D_2 into 25,28(OH)$_2D_2$ and 25,26(OH)$_2D_2$. We first demonstrated that there was no metabolism of 25-OH-D_2 into 25,28(OH)$_2D_2$ in the isolated perfused kidney, as indicated by the absence of a UV-absorbing peak in the migration position of the authentic synthetic standard of 25,28(OH)$_2D_2$ (Figure 1). Later, we also demonstrated that

25,28(OH)$_2$D$_2$ was not a significant circulating metabolite of vitamin D$_2$ in a vitamin D$_2$ intoxicated rat as indicated by the absence of a radioactive peak in the migration position of an authentic synthetic standard of 25,28(OH)$_2$D$_2$ (Figure 5). In the present study as we did not have the synthetic standard of 25,26(OH)$_2$D$_2$, we could not establish the elution position of 25,26(OH)$_2$D$_2$ on our HPLC systems. As a result, we were unable to conclude whether there was any formation of 25,26(OH)$_2$D$_2$ in both our in vitro and our in vivo studies. However, Koszewski et al. (1988) in their recent study definitely established that 25,26(OH)$_2$D$_2$ was not a major circulating metabolite of 25-OH-D$_2$ in rat. Thus, it appears that the preferred substrate for the enzymes responsible for both C-28 and C-26 hydroxylations is 24,25(OH)$_2$D$_2$, but not 25-OH-D$_2$. In our previous study (Reddy & Tserng, 1986) we have demonstrated that 1,25(OH)$_2$D$_2$ is hydroxylated first at C-24 to form 1,24,25(OH)$_3$D$_2$, which is then further hydroxylated either at C-28 to form 1,24,25,28(OH)$_4$D$_2$ or at C-26 to form 1,24,25,26(OH)$_4$D$_2$. Thus, in an analogous fashion, even though we did not have direct evidence for the conversion of 24,25(OH)$_2$D$_2$ into both 24,25,28(OH)$_3$D$_2$ and 24,25,26(OH)$_3$D$_2$, it may be hypothesized that 25-OH-D$_2$ is hydroxylated either at C-28 or at C-26 only after it is hydroxylated first at C-24.

Understanding the pathways of inactivation of both 25-OH-D$_2$ and 24-OH-D$_2$, the two physiologically significant metabolites of vitamin D$_2$, is of importance, especially in hypervitaminosis D$_2$, a condition that is not uncommon in clinical medicine as vitamin D$_2$ is used routinely as a therapeutic agent. In vitamin D$_2$ intoxicated humans the circulating levels of 25-OH-D$_2$ can be as high as 250–750 ng/mL, and hypercalcemia that develops in this clinical situation is being related to the high circulating levels of 25-OH-D$_2$ (Mawer et al., 1985). In a recent study, Koszewski et al. (1988) investigated the metabolism of vitamin D$_2$ in a systematic fashion in hypervitaminosis D$_2$. They have indicated that both 24-OH-D$_2$ and 25-OH-D$_2$ circulate in significant amounts in a vitamin D$_2$ intoxicated rat and that 24-OH-D$_2$ is inactivated through its conversion into 24,26(OH)$_2$D$_2$. Until our present study, the only information that is available regarding the inactivation pathway of 25-OH-D$_2$ is its conversion into 24,25(OH)$_2$D$_2$ (Jones et al., 1979, 1980). Our study further extends the inactivation pathway of 25-OH-D$_2$ by demonstrating its conversion into both 24,25,28(OH)$_3$D$_2$ and 24,25,26(OH)$_3$D$_2$. Thus, the study by Koszewski et al. (1988) and our present study represent significant contributions of vitamin D$_2$ metabolism from the more extensively characterized vitamin D$_3$ metabolism. These newly discovered pathways of side-chain metabolism for both 24-OH-D$_2$ and 25-OH-D$_2$ may form a basis for future studies that may help to understand the reasons for the differences that are known to exist between vitamins D$_2$ and D$_3$ in terms of their bioactivity and toxicity in both avian and mammalian species as described in our previous study (Reddy & Tserng, 1986). Studies to compare the biological activity of the two new metabolites of 25-OH-D$_2$ described in this paper with the previously well-studied further metabolites of 25-OH-D$_3$ in terms of (a) calcium absorption by the gut, (b) calcium mobilization from the bone, and (c) their binding affinity to the vitamin D binding protein are presently in progress in our laboratory.

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Registry No. 25-OH-D$_2$, 21343-40-8; 24,25(OH)$_2$D$_2$, 58050-55-8; 24-OH-D$_2$, 58050-56-9; 24,25,26(OH)$_3$D$_2$, 123992-85-8; 24,25,28-(OH)$_3$D$_2$, 123992-86-9.

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